

Title: A gene related to migraine in man.

Migraine is a frequent paroxysmal neuro-vascular disorder, characterized by recurrent attacks of disabling headache, vomiting, photo/phonophobia, malaise, and other general symptoms (migraine without aura). Up to 20% of 5 patients may, in addition, experience transient neurological (aura) symptoms during attacks (migraine with aura) (HCC, 1988). Up to 24% of females and 12% of males in the general population are affected, however with variable attack frequency, duration and severity (Russell et al., 1995).

10 Knowledge about the mechanisms of the final common pathway of migraine attacks has increased substantially the last five years, resulting in improved, though still only symptomatic (and sub-optimal) acute treatment for the attack. There is, however, still very little knowledge about the etiology of 15 migraine attacks, i.e. why and how attacks begin and recur. Accordingly, prophylactic treatment for migraine is non-specific and has only limited efficacy.

Family, twin and population-based studies suggest that 20 genetic factors are involved in migraine, most likely as part of a multifactorial mechanism (reviewed by Haan et al., 1996). The complex genetics has hampered identification of candidate genes for migraine. Familial Hemiplegic Migraine (FHM) is a rare, autosomal dominant, subtype of migraine with aura, associated with ictal hemiparesis and, in some families 25 cerebellar atrophy (HCC, 1988). Otherwise, the symptoms of the headache and aura phase of FHM and "normal" migraine attacks are very similar and both types of attacks may alternate within subject and co-occur within families. FHM is thus part of the migraine spectrum and can be used as a model 30 to study the complex genetics of the more common forms of migraine (Haan et al., 1996). A gene for FHM has been assigned to chromosome 19p13 in about half of the families tested (Joutel et al., 1993; Ophoff et al., 1994;

Joutel *et al.*, 1995). Remarkably, cerebellar atrophy was found only in families with FHM linked to chromosome 19p13, but not in unlinked families. Recently, we showed the 19p13 FHM locus to be also involved in "normal" migraine
5 (May *et al.*, 1995).

Episodic ataxia type 2 (EA-2) is another, autosomal dominant, paroxysmal neurological disorder, characterized by acetazolamide-responsive attacks of cerebellar ataxia and migraine-like symptoms, and interictal nystagmus and
10 cerebellar atrophy. Recently, a gene for EA-2 was assigned to chromosome 19p13, within the same interval as for FHM (Kramer *et al.*, 1995). This finding, as well as the clinical similarities, raise the possibility of EA-2 and FHM being allelic disorders.

15 Since other hereditary episodic neurological disorders responding to acetazolamide (such as hypokalaemic and hyperkalaemic periodic paralysis), as well as EA type-1 (which, in contrast to EA-2, is associated with continuous myokymia and non-responsive to acetazolamide) have all been
20 associated with mutations in genes encoding for ion channels (Ptacek *et al.*, 1991; Ptacek *et al.*, 1994; Brown *et al.*, 1994), we specifically looked for similar genes within the FHM and EA-2 candidate region.

In view of the above, the FHM/EA-2 locus can, since FHM
25 is part of the migraine spectrum, thus be used to study the genetic factors and biological mechanisms that are related to various episodic neurological disorders such as FHM, EA-2, common migraine and others such as epilepsy.

Calcium channels are multisubunit complexes composed of
30 at least an α_1 , an $\alpha_2\delta$, and a β subunit. The central α_1 subunit is functionally the most important component, acting as a voltage sensor and forming the ion-conducting pore. The other subunits have auxiliary regulatory roles. The membrane topology of the α_1 subunit consists of four hydrophobic motifs
35 (I to IV), each containing six transmembrane α -helices (S1-

S6) and one hairpin (P) between S5-S6 that spans only the outer part of the transmembrane region.

The present invention provides an isolated and/or recombinant nucleic acid, or fragments thereof, encoding a 5 Ca^{2+} -channel $\alpha 1$ subunit related to familial hemiplegic migraine and/or episodic ataxia type-2, derived from a gene present on chromosome 19p13.1-19p13.2; a gene encoding the $\alpha 1$ (ion-conducting) subunit of a P/Q-type voltage gated calcium channel. The present invention also provides access to and 10 methods to study the genetic background and identify other subunits of the calcium channel subunit complexes and the proteins related therewith that are associated with the genetic factors and biological mechanisms that are related to various episodic neurological disorders such as FHM, EA-2, 15 common migraine and others such as epilepsy which are related to cation channel dysfunction.

The sequence of the cDNA of the gene is highly related (>90%) to a brain-specific rabbit and rat voltage gated P/Q-type calcium channel $\alpha 1$ subunit (Mori et al., 1991; 20 Starr et al., 1991), and the open reading frame consists of 2261 amino acid residues. Northern blot analysis showed a brain-specific expression, especially in the cerebellum. Primary study of a cosmid contig harbouring the gene already indicated an exon distribution over at least 300 kb of 25 genomic DNA. Recently, a neuronal Ca^{2+} $\alpha 1A$ subunit gene was localized to chromosome 19p13.1-p13.2 by FISH analysis (Diriong et al., 1995). The gene symbol is CACNL1A4 and the $\alpha 1$ subunit is classified as a P/Q-type. No sequence data for the CACNL1A4 gene have been provided by Diriong or others, 30 but the same localization (chromosome 19p13.1) and the identical classification (P/Q-type) suggests that the Ca^{2+} channel $\alpha 1$ subunit we have identified is very similar to CACNL1A4. No relation with migraine has been disclosed for CACNL1A4. The genomic structures of three other human Ca^{2+} 35 channel $\alpha 1$ subunit genes (CACNL1A1, CACNL1A2 and CACNL1A3) have been published to date (Hogan et al., 1994; Soldatov, 1994; Yamada et al., 1995). Both CACNL1A1 and CACNL1A2 span

about 150 kb and consist of 50 and 49 exons, respectively. The smaller CACNL1A3 gene is composed of 44 exons, distributed over 90 kb.

The present invention also provides an isolated and/or recombinant nucleic acid comprising alleles of the invented gene which contain mutations relevant to the occurrence of migraine and other neurological disorders which are related to cation channel dysfunction. Such mutations are for example a mutation at codon 192 resulting in the replacement of arginine by glutamine (R192Q), and/or a mutation at codon 666 resulting in the replacement of threonine by methionine, and/or a mutation at codon 714 resulting in a replacement of valine by alanine and/or a mutation at codon 1811 resulting in a replacement of isoleucine by leucine, but also other mutations of alleles of said gene which bear relationships with cation channel dysfunction.

The present invention also provides isolated and/or recombinant nucleic acid comprising alleles of said gene which contain a polymorphic CA-repeat sequence specific for various alleles of said gene. The present invention also provides isolated and/or recombinant nucleic acids comprising alleles of said gene which contain a CAG repeat.

The present invention also provides methods and tests (such as PCR, but also other tests to detect or amplify nucleic acids are known in the art) to detect, identify and localize or distinguish genes and alleles of such genes, or fragments thereof, encoding for proteins or α , β or γ sub-units of specific cerebral cation channels, more specifically the invented gene and its various alleles encoding the $\alpha 1$ subunit of a P/Q-type voltage gated calcium channel and the gene encoding the $\beta 2$ sub-unit, which are involved in the primary pathogenesis of neurological disorders such as FHM, migraine, EA-2 and SCA6. With such methods and tests one can study abnormalities of said gene.

The invention also provides recombinant expression vectors comprising isolated and/or recombinant nucleic acid comprising alleles of said genes or fragments therof, and

provides host cells or animals that comprise such vectors or that are otherwise transformed with an isolated and/or recombinant nucleic acid according to the invention.

The invention thus also provides a rationale and methods 5 for the testing and the development of specific prophylactic medication for migraine and other episodic neurological, in particular brain, disorders, such as epilepsy, associated with cation channel dysfunction.

The invention for example provides cells or animals that 10 comprise recombinant vectors that comprise variants of said genes or cells or animals that are transformed with said variants. Also, the invention provides means to identify naturally occurring variants of experimental animals with changes in said gene related to FHM, EA-2, SCA7, migraine or 15 other neurological disorders associated with cation channel dysfunction. An example of such an animal is the tottering mouse, and its variants called leaner and rolling, described in the experimental part of the invention. The invention also provides cells or animals in which changes such as deletions 20 or mutations in said gene have been introduced by recombinant nucleic acid techniques. All such cells or animals provided by the invention can be used to study the pathophysiology of FHM, EA-2, migraine or other neurological disorders associated with cation channel dysfunction, for example to 25 test or develop specific medication for the treatment of said disorders.

The invention also provides proteins or peptides encoded by said genes, or fragments thereof, related with cation channel dysfunction, and detection of such proteins or 30 peptides by antibodies directed against said proteins or peptides. Such antibodies can be of natural or synthetic origin, and can be produced by methods known in the art. Such proteins and antibodies and detection methods can be used to further *in vitro* or *in vivo* studies towards the 35 pathophysiology of FHM, EA-2, migraine or other neurological disorders associated with cation channel dysfunction, in addition such proteins, antibodies and detection methods can

also be used to diagnose or identify such disorders in patients or in experimental animals.

Experimental Procedures

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Subjects

Sixteen FHM patients were selected, including eight individuals from four unrelated chromosome 19-linked FHM families (*NL-A*, *UK-B*, *USA-C* (Ophoff *et al.*, 1994), and *USA-P* (Elliot *et al.*, 1995), two affected individuals from two small FHM families from Italy (*Italy I & II*) and six individuals with sporadic hemiplegic migraine (i.e. no other family member was shown to suffer from attacks of hemiplegic migraine). In families *NL-A* and *USA-P* cerebellar ataxia and/or nystagmus is associated with FHM. An additional set of four subjects from four unrelated EA-2 families linked to chromosome 19, was also included (CAN-25, -45, -191, -197). Fifty randomly collected individuals from the Dutch population (Smith *et al.*, 1988) were used as a control to determine the allele frequencies of polymorphic sites.

Patients with migraine with or without aura were diagnosed according to the international Headache Society (IHS) classification criteria. Patients attending the neurology outpatient clinic of Leiden University Medical Center, The Netherlands, and patients responding to calls in local newspapers or in the periodical of the Dutch Migraine Patients Association, were screened for a positive family history of migraine. Only families with migraine in at least two generations were asked to participate. Proband (n=36) and relatives (n=492) were personally examined and interviewed using semi-structured questionnaires. The questionnaire included questions about age at onset, frequency and duration of attacks, aura symptoms, premonitory signs and symptoms, triggers for attacks, medication, and additional headaches. When family members were not available for a personal interview, information on their migraine was collected by interviewing their relatives. Because of the low

validity of diagnosing migraine auras through relatives, we only assessed the presence or absence of migraine headaches. Whenever possible, medical records were examined.

5 **Genomic structure**

Ten different cosmids from the contig extending the invented gene, were subcloned separately in either M13 or pBlueScript KS vector. From each cosmid library at least 3x96 random clones with an average insert size of about 2 kb, were 10 picked. Positive clones were identified by hybridization techniques and subsequently sequenced with vector-specific primers; intron-exon boundary sequences were completed using cDNA-based primers.

15 **Mutation analysis, DHPLC and SSCP**

Genomic DNA was used as template to generate polymerase chain reaction (PCR) products for single-strand conformational polymorphism (SSCP) analysis and denaturing high-performance liquid chromatography (DHPLC).
20 Amplifications were performed in standard conditions with primer pairs as listed in Table 1 or listed below. Except for the 5' side of exon 6, primers were chosen to produce fragments that contained a single exon and at least 35 basepairs (including primer) of each flanking intron sequence.
25 Amplification of exons 1 and 20 was performed producing two overlapping fragments and exon 19 was amplified into three overlapping fragments. In addition, the following markers;

D10S191 Primer sequence 1 CTT TAA TTG CCC TGT CTT C
30 Primer sequence 2 TTA ATT CGA CCA CTT CCC

D10S245 Primer sequence 1 AGT GAG ACT CGT CTC TAA TG
 Primer sequence 2 ACC TAC CTG AAT TCC TGA CC

35 D10S89 Primer sequence 1 AAC ACT AGT GAC ATT ATT TTC A
 Primer sequence 2 AGC TAG GCC TGA AGG CTT CT

DHPLC (Oefner *et al.*, 1995; Hayward *et al.*, 1996) was carried out on automated HPLC instrumentation. Crude PCR products, which had been subjected to an additional 3-minute 95°C denaturing step followed by gradual reannealing from 5 95-65°C over a period of 30 minutes prior to analysis, were eluted with a linear acetonitrile (9017-03, J.T. Baker, Phillipsburg, N.J., USA) gradient of 1.8% per minute at a flow-rate of 0.9 ml/min. The start- and end-points of the gradient were adjusted according to the size of the PCR 10 products (Huber *et al.*, 1995). The temperature required for successful resolution of heteroduplex molecules was determined empirically by injecting one PCR product of each exon at increasing mobile phase temperatures until a significant decrease in retention was observed.

15 For SSCP analysis, primary PCR products were labeled by incorporation of [α -³²P]dCTP in a second round of PCR. Samples were diluted and denatured in formamide buffer before electrophoresis. SSCP was carried out according to published protocols (Orita *et al.*, 1989; Glavac *et al.*, 1994). 20 Digestion of several exons to yield products suitable for SSCP analysis.

Sequencing of PCR products was performed with an ABI 377 automated sequencing apparatus with cycle sequencing according to the manufacturer. Furthermore, PCR products were 25 cloned in the TA vector (Invitrogen) and subjected to manual dideoxy sequence analysis (T7 Sequencing kit, Pharmacia Biotech.).

A total of 481 blood samples were collected from patients with migraine. Genomic DNA was isolated as described 30 by Miller *et al.*, 1988. The analyses of the highly informative microsatellite markers D19S391, D19S394, D19S221 and D19S226, D10S191, D10S245 and D10S89 were performed by PCR; primer sequences related to these markers are available through the human Genome Data Base (GDB).

35 The relative frequencies of marker alleles were estimated on the entire family material, with the relevant

correction for genetic relationships between individuals (Boehnke, M, 1991) with the ILINK option of the I-INKAGE package, version 5.03 (Lathrop et al., 1985). The following marker order and recombination frequencies were used in the 5 multipoint sib-pair analysis: D19S391-5%-D19S394-3%-D19S221-5%-D19S226. Affected-sib-pair analysis was performed using the MAPMAKER/SIBS software package, simultaneously including marker information for all four DNA markers (Kruglyak, 1995). Separate analyses were performed for migraine with aura, 10 migraine without aura, and a combination of both. Allowance was made for dominance variance. When more than two affected sibs occurred in a single sibship, weighted scores were computed according to Suarez and Hodge (1979).

In a sib-pair analysis, the occurrence of parental 15 marker alleles is compared among sibs. Normally, 25% of sib pairs share their marker alleles from both parents, 50% share one marker allele from one of their parents, while the remaining 25% share no parental allele. Deviations from this pattern towards increased sharing, and consistent with the 20 constraints of Holmans's (1993) possible triangle, are explained as linkage (expressed as the maximum lod score MLS). Increased sharing of marker alleles thus indicate that the marker is located closely near a genetic risk factor. The 25 relative-risk ratio for a sib (λ_R), defined as the ratio of the prevalence of a disease in sibs of affected individuals, divided by the prevalence of a disease in the population, can be calculated (May et al., 1995). In other words:

$$\lambda_R = \frac{\text{Affected risk for sib of a proband}}{\text{Affection risk for an individual in the general population}}$$

Results

35 Genomic structure

The combination of hybridization and PCR strategies resulted in a rapid assembly of the complete coding sequence

of the human cDNA, with an open reading frame of 6783 nucleotides encoding 2261 amino acid residues (figure 4). The spatial distribution of the human Ca^{2+} channel expression was assayed in rhesus monkey tissues. Total RNA was isolated from 5 several tissues, including various brain structures, and probed with a human cDNA fragment. The probe detected a major transcript of approximately 9.8 kb in cerebellum, cerebral cortex, thalamus and hypothalamus, whereas no transcript was detected in heart, kidney, liver or muscle. There was also no 10 hybridization signal found in RNA preparations from mouse skin tissue or from human peripheral lymphocytes. In addition, an attempt to amplify parts of the cDNA from human peripheral lymphocytes failed.

Complete alignment between the cDNA and individual exon 15 sequences was achieved, allowing the establishment of the exon-intron structure (Table 1). The reconstruction of the exon-intron structure of the CACNL1A4 gene revealed 47 exons ranging in size from 36 bp (exon 44) to 810 bp (exon 19). The exons are distributed over about 300 kb at genomic DNA level. 20 The result shows that the first 10 exons are located in a region of about 150 kb covered by the first 5 cosmids of the contig indicating relatively large introns at 5' side of the gene. Sequences (Figure 1) were obtained of all exons including approximately 100 bp of flanking introns, except 25 for intron 5 adjacent to exon 6. The forward primer of exon 6 harbours the splice junction and 3 bp of exon 6. Splice sites around all exons are compatible with consensus sequence with the exception of splice donor and acceptor of the first intron.

30 The cosmid contig that yielded the initial Ca^{2+} channel gene exons was extended to cover more than 300 kb. Hybridization experiments showed that the first and last cosmids of the contig were positive for 3'- and 5'-end cDNA sequences, respectively, indicating a genomic distribution of 35 the gene over at least 300 kb (Figure 2). The cosmid contig has been placed into the LLNL physical map of chromosome 19 at band p13.1, between the markers D19S221 and D19S226

(Figure 2). We identified a new polymorphic CA-repeat sequence (D19S1150) on the cosmid contig. Oligonucleotide primers (Table 1) flanking the repeat were synthesized and amplification was performed by PCR as described. Analysis of 5 D19S1150 in 45 random individuals from the Dutch population revealed nine alleles with an observed heterozygosity of 0.82. This highly polymorphic marker is located within the gene and is therefore very useful in genetic analysis.

10 **Mutation analysis**

Exons and flanking intron sequences, containing the complete coding region of CACNL1A4 and part of untranslated sequences, were screened for the presence of mutations by SSCP and DHPLC analysis in 20 individuals with either FHM or 15 EA-2. Several synonymous nucleotide substitutions and polymorphisms were identified including a highly polymorphic (CAG)n-repeat in the 3' untranslated region of exon 47 (Table 2). Of all polymorphisms only one was identified predicting an amino acid change, an alanine to threonine 20 substitution at codon 454 (A454T).

Four different missense mutations were found in FHM patients of which one mutation was observed in two unrelated FHM affected individuals (Table 3). The mutations were shown to segregate with the disease within the families; and were 25 not present in about 100 control chromosomes. A G-to-A transition was identified in family Italy-II at codon 192, resulting in a substitution of arginine to glutamine (R192Q) within the first voltage sensor domain (IS4). A second missense mutation occurs at codon 666, within the P-segment 30 of the second repeat replacing a threonine residue for methionine (T666M) in family USA-P. Two other mutations are located in the 6th transmembrane spanning segment of respectively repeat II and IV. The IIS6 mutation is a T-to-C transition at codon 714, resulting in a substitution of 35 valine to alanine (V714A), identified in FHM family UK-B. The mutation in domain IVS6 is an A-to-C transversion at codon 1811 resulting in a substitution of isoleucine to leucine

(I1811L). This I1811L mutation is found in family *NL-A* and family *USA-C*, two unrelated FHM families. Comparison of haplotypes in this region, including intragenic markers, did not reveal any evidence for a common founder of family *NL-A* and *USA-C* (data not shown). No mutation was found in FHM family *Italy-I* nor in the six sporadic hemiplegic migraine patients. In addition to missense mutations in FHM families, we also identified mutations in two out of four EA-2 families (Table 3). In EA-2 family *CAN-191*, a basepair deletion occurs in exon 22 at nucleotide position 4073 causing a frameshift and a premature stop. The second EA-2 mutation is a transition of G-to-A of the first nucleotide of intron 24, predicted to leading to an aberrant splicing in family *CAN-26*. The invented gene also contains a CAG repeat, of which expansions have been found in patients with autosomal dominant cerebellar ataxia (SCA6). Hence FHM, EA-2 and SCA6 are allelic ion channel disorders and different mutations are associated with different clinical symptomalogies.

Our study patients with common migraine (with or without aura) included 36 independent multigenerational Dutch families. At least some data were available on 937 family members and 212 persons who "married-in" (spouses). Of these, 442 family members (247 affected) and 86 spouses (7 affected) were personally interviewed. The distribution of the different types of migraine among the 247 affected family members are as follows: 132 family members showed migraine without aura, 93 showed migraine with aura and 22 showed months-migraine, not fulfilling all critera by IHS. Among the 7 affected spouses these figures were 4, 1 and 2, respectively. We scored the parental transmission of migraine in the 36 families (Tabel 4) to investigate if an additional X-linked dominant or mitochondrial gene was involved. An approximately 2.5:1 preponderance of females among the migraine sufferers was noted, which remained in the affected offspring. Affected fathers were found to transmit migraine

to their sons in 21 cases, making X-linked dominant or mitochondrial inheritance highly unlikely.

The genetic analysis included 204 potentially affected sib pairs; after correction for more than one sib pair in a single sibship the total number of sib pairs was 108. Affected-sib-pair analysis was performed for sib pairs who were both affected with any form of migraine and, in separate analyses, for sib pairs who where both suffering from either migraine with aura or migraine without aura. The informativeness of the region between the markers D19S391, D19S394, D19S221 and D19S226 varied between 82% and 96%. The combined analysis of migraine with and without aura resulted in a maximum multipoint lod score of 1.69 ($p \approx 0.005$) with marker D19S226. For migraine with aura the maximum multipoint lod score was 1.29 corresponding with $p \approx 0.013$ with marker D19S394. The maximum lod score for migraine without aura was not significant (MLS < 0.25) (data not shown). The relative risk ratio for a sib to suffer from migraine with aura (λ_s), defined as the increase in risk of the trait attributable to the 19p13 locus, varied between $\lambda_s = 1.5$ (for marker D19S394) and $\lambda_s = 2.4$ (for marker D19S226). When combining migraine with and without aura, λ_s was 1.25. In a selected portion of 36 Dutch families with migraine with aura and without aura, affected sib-pair analysis was performed for sib pairs who were affected with any form of migraine. The following markers, flanking the $\beta 2$ (CACNB2) calcium channel subunit gene on chromosome 10p12, were tested: D10S191, D10S246 and D10S89. For the combined phenotype (migraine with and without aura) a maximum multipoint lod score of 0.95 ($p < 0.01$) was obtained with marker; D10S191. This result gives independent evidence for a role of the P/Q type Ca^{2+} channel in migraine and other neurological disorders.

Discussion

The genomic structure of the exemplified invented gene revealed 47 exons distributed over about 300 kb (Table 1; Figure 1). A comparison of the gene structure to already

known Ca^{2+} channel $\alpha 1$ subunit genes (CACNL1A1, CACNL1A2, and CACNL1A3) (Soldatov, 1994; Yamada *et al.*, 1995; Hogan *et al.*, 1995), reveals a similar number of exons (50, 49, and 44 respectively) but a larger genomic span (300 kb vs 5 90-150 kb). Remarkably, all splice sites are according to consensus sequence except for intron 1. Splice donor as well as splice acceptor of the first intron do not contain the expected gt...ag intron sequence. An incorrect cDNA sequence is unlikely because the cDNA sequence containing the junction 10 of the first two exons is identical to rabbit and rat sequence. Sequences corresponding to splice donor and acceptor are present in exon 1 and 2, suggesting an additional (yet unidentified) exon in the first intron encompassing part of sequences of exon 1 and exon 2.

15 To test the possible involvement of the invented gene relating to the Ca^{2+} -channel sub-unit in migraine FHM, SCA6 and EA-2, we performed a mutation analysis by DHPLC and SSCP and found several alterations (For example Table 2 & 3). Only one missense variation was observed also present in 2% of the 20 normal controls (Table 2). This polymorphism is a alanine to threonine substitution at codon 454 (A454T), located in the intracellular loop between IS6 and IIS1 (Figure 2). This region contains a conserved alpha interaction domain (AID) that binds subunits (De Waard *et al.*, 1996). However, A454T 25 is located outside the AID consensus sequence and is not likely to be involved.

The identification of two mutations that disrupt the predicted translation product of the invented gene in two unrelated EA-2 patients and the segregation of these 30 mutations with the episodic ataxia phenotype in their families provide strong evidence that the invented gene is the EA-2 gene. A basepair deletion leads to a frame-shift in the putative translation product and encounters a stop codon in the next exon. The frame-shift in this EA-2 family is 35 predicted to yield a calcium channel $\alpha 1$ subunit polypeptide consisting of repeat I and II, and a small portion of repeat III (IIS1). The G-to-A transition of the first nucleotide of

intron 24 is affecting the nearly invariant GT dinucleotide of the intronic 5' splice junction. The brain-specific expression of the exemplified invented gene makes it extremely difficult to test the hypothesis that this mutation 5 produces aberrantly spliced RNAs by retaining the intron or utilizing other cryptic 5' splice sites.

The frameshift and splice site mutations in EA-2 may suggest a dominant negative effect of the truncated proteins by overruling the (corresponding) intact $\alpha 1$ subunits.

10 No mutations were found in the remaining EA-2 families (CAN-25 and -197). The use of two independent techniques for mutation screening (DHPLC and SSCP) makes it unlikely that we missed a heterozygote PCR product. Mutations in the promoter region or in intron sequences, resulting in aberrant 15 splicing, may have been the cause of EA-2 in these families. We could also have missed a mutation around the splice acceptor site of intron 5, covered by the forward primer of exon 6. However, larger deletions of e.g. complete exons with flanking intron sequence will disturb the predicted 20 translation product, like the ΔC_{4073} and splice site mutation do, but this is not detectable by a PCR-based screening method but can be seen Southern blot analysis instead.

Four different missense mutations were identified in five unrelated FHM families. These mutations all segregate 25 with FHM within a family and are not observed in over 100 normal chromosomes. The first missense mutation that we describe in the exemplified invented gene occurs in the IS4 domain of the $\alpha 1$ subunit (Table 3; Figure 3). The S4 domains are postulated to be voltage sensors because they have an 30 unusual pattern of positively charged residues at every third or fourth position separated by hydrophobic residues (Tanabe et al., 1987). In calcium channels the positively charged amino acid is an arginine residue (Stea et al., 1995). The mutation in FHM family Italy-II predicts a substitution of 35 the first arginine in the IS4 segment with a neutral, non-polar glutamine (R192Q). The change of the net positive

charge of this conserved region of the protein may influence correct functioning of the voltage sensor.

The second missense mutation in FHM family *USA-P* occurs in the P-segment of the second transmembrane repeat. A C-to-T transition predicts substitution of a threonine residue with methionine at codon 666 (T666M). Various observations have shown that P-segments, the hairpin between S5 and S6 that spans only the outer part of the transmembrane region, form the ion-selectivity filter of the pore and binding sites for toxins (Guy and Durell (1996)). Alignment of protein sequence of different P-segments indicating that some residues occur in many different channel genes (Guy and Durell, 1996). The T666M substitution alters one of the conserved residues in the P-segment. It is conceivable that an alteration of a P-segment affects the ion-selectivity or toxin binding of a channel gene.

The remaining two missense mutations identified in FHM families alter the S6 segment of the second and the fourth repeat. A valine to alanine substitution in FHM family *UK-B* is found in domain IIS6 at codon 714 (V714A). Domain IVS6 is mutated in two unrelated FHM families (*NL-A* and *USA-C*), predicting a isoleucine to leucine substitution at codon 1811 (I1811L). The V714A and I1811L missense mutations do not really change the neutral-polar nature of the amino acid residues. However, both S6 mutations are located nearly at the same residue at the intracellular site of the segment and are conserved in all calcium channel $\alpha 1$ subunit genes. In addition, the A-to-C transversion leading the I1811L substitution occurred in two unrelated FHM families on different haplotypes indicating recurrent mutations rather than a founder effect. Although the exact function of the S6 domains are not known, these data strongly suggest that mutations in IIS6 and IVS6 result in FHM.

The I1811L mutation is present in two FHM families of which one (*NL-A*) also displays a cerebellar atrophy in (some) affected family members. The presence of cerebellar atrophy in FHM families has been reported in about 40% of chromosome

19-linked FHM families, whereas none of the unlinked families was found to have cerebellar atrophy (Terwindt et al., 1996).

The I1811L mutation excludes the possibility of allelic mutations in FHM and FHM with cerebellar atrophy. However, it 5 is likely that FHM or FHM with cerebellar atrophy are the result of pleiotropic expression of a single defective gene.

No mutation was found in a small Italian FHM family (Italy-I). Apart from the possibilities discussed for EA-2, it should be noted that linkage to 19p13 was only suggested 10 but never proved with significant lod scores (M. Ferrari, personal knowledge).

The four missense mutations identified indicate a mechanism for FHM in which both alleles of the $\alpha 1$ subunit are expressed, one harbouring an amino acid substitution which 15 affects the function of this calcium channel $\alpha 1$ subunit by reducing or enhancing the electrical excitability. The relationship of FHM and other types of migraine makes it highly rewarding to investigate the involvement of the only missense variant observed (A454T) (Table 2), and to continue 20 the search for other variants of the exemplified invented gene specific for common types of migraine.

The mutations in EA-2 and FHM demonstrate among others that the brain specific calcium channel gene CACNL1A4 is responsible for both EA-2 and FHM, and is also involved in 25 the primary pathogenesis of the more common forms of migraine. We conducted the common migraine study in an independent sample of 36 extended Dutch families, with migraine with aura and migraine without aura. We found significant increased sharing of the marker alleles in sibs 30 with migraine with aura (MLS=1.29 corresponding with $p \approx 0.013$). Although no such increased sharing was found for migraine without aura, a combined analysis for both migraine types resulted in an even more significant increased sharing (MLS=1.69 corresponding with $p \approx 0.005$). These results clearly 35 indicate the involvement of the calcium α_{1A} -subunit gene region on 19p13 in both migraine with and without aura; the contribution to migraine with aura, however, seems strongest.

The positive findings in our study clearly demonstrate an involvement of the FHM locus region in non-hemiplegic familial migraine, notably in migraine with aura. The P/Q-type calcium channel α_{1A} -subunit gene on chromosome 5 19p13 may be an "aura-gene" and is involved in both FHM and migraine with aura, but not in migraine without aura. This however, seems unlikely since an increased sharing of marker alleles was also found when we combined the results for migraine with and without aura. Furthermore, the increase in 10 sharing was stronger than expected to be only due to the contribution of migraine with aura. An alternative explanation is that the gene is involved in both types of migraine, but in migraine without aura there is an additional strong effect of other, possibly environmental factors, 15 thereby reducing the penetrance.

The latter view is also supported by the results obtained from calculating the relative risk ratios (λ_R) for sibs from affected individuals to also have migraine. The relative risk ratio for a sib to suffer from migraine with 20 aura was $\lambda_R=2.4$. When combining migraine with and without aura, λ_R was 1.25. In a population-based study the relative risk for first degree relatives of probands with migraine with aura to also have migraine with aura was $\lambda_R=3.8$. Because of the female preponderance among migraine patients, X-linked 25 dominant or mitochondrial inheritance has been suggested to be involved in familial migraine. Although a predominant maternal inheritance pattern was noted in our families, X-linked dominant or mitochondrial inheritance were found to be highly unlikely because affected fathers transmit migraine to 30 their sons. Furthermore, the predominant maternal inheritance can be explained by the female preponderance among the migraine patients.

We conclude that the well-established genetic contribution to the etiology of migraine is partly, but not 35 entirely, due to genetic factors located in the chromosomal region of the P/Q-type calcium channel α_{1A} -subunit gene. Further analysis of the cerebral distribution and function of

this calcium channel, as well as of the "mutated channels", will help to unravel the pathogenetic pathway of migraine. It may also contribute to a better understanding of the mechanisms involved in related disorders such as episodic ataxia type-2, autosomal dominant cerebellar ataxia (SCA6), cerebral atrophy, and epilepsy, which all have been found to be associated with mutations in this gene. Study of FHM, EA-2 mutants and variants such as the A454T variant expressed in vitro or in mouse or other experimental animal models will ultimately lead to better understanding of the diseases, their cellular mechanisms, and the clinical relationship between FHM, EA-2, migraine, and other episodic neurological disorders such as epilepsy, and will provide rationales for the development of prophylactic therapy.

15

Localization and identification of the mouse gene related to the neurological mouse mutations tottering, leaning and rolling.

The tottering (tg) mutation arose spontaneously in the DBA inbred strain, and has been back-crossed into a C57BL/6J (B6) inbred strain for at least 30 generations. The genome of the tg mouse therefore is of B6 origin except for a small region around the tg gene on chromosome 8. Interestingly, the chromosome 8 region in mouse has synteny with the human chromosome 19p13.1, in which the human calcium channel alpha1 subunit has been identified. We therefore consider the tg locus as a possible site of the mouse homologue of the human calcium channel gene.

To determine the exact localization of the mouse homologue, PCR was carried out with primers based on human cDNA sequence selected from Figure 1 and mouse genomic DNA as template. In human, primers were known to be located in different flanking exons. PCR amplification on human DNA yielded a 1.5kb fragment.

30 Forward primer : 5'- caa cat cat gct ttc ctg cc-3'
Reversed primer: 5'- atg atg acg gcg aca aag ag-3'

Amplification on mouse DNA yielded a 750-bp fragment. The fragment mainly consists of intronic sequences. SSCP analysis revealed several polymorphisms in the different inbred strains (each strain a specific pattern). Analysis of 5 amplified product of the tg/tg (homozygote) and tg/+ (heterozygote) mice demonstrated a DBA specific signal in the tg/tg mouse, and a heterozygous pattern of DBA and B6 inbred strains in the heterozygous tg/+ mouse.

These results show that the mouse homologue of the human 10 calcium channel alpha1 subunit is located within the mouse tottering interval on chromosome 8.

In conclusion: the phenotypic characteristics of the tg mouse (tg/tg and tg/+) suggest involvement of ion-channels in the tg-etiiology. The localization of the mouse homologue of 15 the human calcium gene within the tottering interval show that a tottering phenotype in mouse is caused by a mutation in the mouse homologue of the CACNL1A4 gene. With various variants of the tottering mouse (the Jackson Laboratory, Bar Habor, ME, USA), such as the leaner and rolling varieties, 20 such mutations in the mouse homologue of the CACNL1A4 gene can be found, clearly demonstrating that the gene is related to a variety of episodic neurologic disorders and using this genetic information one can engage in a variety of pathophysiological studies, as for example indicated below.

25 The tg mutation arose spontaneously in the DBA/2 inbred strain. tg/tg homozygotes are characterized by a wobbly gait affecting the hindquarters in particular, which begins at about 3 to 4 weeks of age, and by intermittent spontaneous seizures which resemble human epileptic absence seizures. The 30 central nervous system of the mice appears normal by light microscopy. There is no discernible cerebellar hypoplasia. In fluorescent histochemistry studies tg/tg mice show a marked increase in number of noradrenergic fibers in the terminal fields innervated by locus ceruleus axons, the hippocampus, 35 cerebellum, and dorsal lateral geniculate. Treatment of neonatal tg/tg mice with 6-hydroxydopamine, which selectively causes degeneration of distal noradrenergic axons from the

locus ceruleus, almost completely abolishes the ataxic and seizure symptoms.

The leaner mutation of the tottering mouse arose spontaneously in the AKR/J strain. Homozygotes are recognized 5 at 8 to 10 days of age by ataxia, stiffness, and retarded motor activity. Adults are characterized by instability of the trunk, and hypertonia of trunk and limb muscles. The cerebellum is reduced in size, particularly in the anterior region, in tg<la>/tg<la> mice, as is the case with a certain 10 number of FHM patients. There is loss of granule cells beginning at 10 days of age and loss of Purkinje and Golgi cells beginning after 1 month. Cell loss later slows but continues throughout life. Granule and Purkinje cells are more severely affected than Golgi cells and the anterior 15 folia more severely affected than other parts of the cerebellum. The cerebellum of tg<la>/tg mice shows shrinkage and degenerative changes of the Purkinje cells. The loss in cerebellar volume in tg<la>/tg and in tg/tg mice is specific to the molecular layer, with no change in volume of the 20 granule cell layer or the white matter layer. Allelism of leaner with tottering was shown in complementation and linkage tests.

A third variety of the tottering mouse is (tg<rol>) called the rolling Nagoya. Found among descendants of a cross 25 between the SIII and C57BL/6 strains, the tg<rol> mutation apparently occurred in the SIII strain. Homozygotes show poor motor coordination of hindlimbs that may lead to falling and rolling, and sometimes show stiffness of the hindlimbs and tail. No seizures have been observed. Symptoms are 30 recognizable at 10 to 14 days old. They appear a little earlier than those of tg/tg mice and are somewhat more severe. The cerebellum is grossly normal until 10 days of age, but after that grows more slowly than normal. The size 35 of the anterior part of the central lobe of the cerebellum is reduced with reduction in the numbers of granule, basket, and stellate cells but normal numbers of Purkinje cells. There is a reduced concentration of glutamate and an increasd

concentration of glycine and taurine in the cerebellum and decreased activity of tyrosine hydroxylase in the cerebellum and other areas.

Legends to figures**Figure 1**

Nucleic acid sequences of 47 exons and flanking intron
5 sequences containing the complete coding region of the
invented gene and part of untranslated sequences.

Figure 2

Genetic map, cosmid contig and global exon distribution of
10 the invented gene on chromosome 19p13.1. The cosmid contig is
shown with EcoRI restriction sites, available via Lawrence
Livermore National Laboratory; exon positions are indicated
schematically, regardless of exon or intron sizes (Table 1).
D19S1150 is a highly polymorphic intragenic (Ca)_n-repeat.
15

Figure 3

Membrane topology of $\alpha 1$ subunit of the P/Q-type Ca^{2+} -channel.
The location and amino acid substitutions are indicated for
mutations that cause FHM or EA-2

20

Figure 4

The coding sequence of human cDNA of the invented gene with
an open reading frame encoding 2261 amino acid residues.

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- 30

Exon/intron organization of the human invented gene
and exon-specific primer pairs

Table 1

<i>Exon</i>	<i>cDNA</i>	<i>Size</i>	<i>Domain</i>	<i>Cosmid(s)</i>	<i>Primer Forward</i>	<i>Primer Reversed</i>	<i>Size</i>
1	UTR - 568	500		25960 / 30151	tct ccg cag tcg tag ctc ca ccg aaa gga tgt aca agc ag	ggg tgg aga gtg cca tgg tc att ccc aag cct cca ggg tag	320 370
2	569 - 674	106	I S1	30151	cac ctc caa cac cct tct tt	tct gtg ccc tgc tcc act c	240
3	675 - 814	140	I S2, I S3	30151	acg ctg acc ttg cct tct ct	caa cca aaa gcc tcg taa tc	230
4	815 - 906	92	I S3, I S4	28913	aaa acc cac cct ctg ttc tc	ttg tca ggg tcg gaa act ca	160
5	907 - 1059	153	I S5	28913 / 27415	ctt ggt ggc ggg gtt t	ctg cct aat cct ccc aag ag	290
6	1060 - 1253	194		27415	tcc ctt ccc ttg tgt aca tg	gtg ggg ctg tgg tgt cct t	350
7	1254 - 1357	104	I S6	27415	gac aga gcc aca aga gaa cc	agc aaa gag gag tga gtg gg	250
8	1358 - 1473	116		34077 / 27415	ata ctc tgg ctt ttc tat gc	gca tga ctc tct ttg tac tc	230
9	1474 - 1530	57		34077	gca gag aat ggg ggt gg	ctg agg tgg gtt tag agc ag	180
10	1531 - 1623	93		34077	ggg taa cgt ctt ttg ctc ttg c	atg tct ctt ggg cga tag gt	200
11	1624 - 1833	210	II S1	16894 / 32236	att tct tct gaa gga aca gc	gga ggg atc agg gag ttg gc	310
12	1834 - 1946	113	II S2, II S3	16894	caa gcc taa cct cct ctc tg	tca ttc cag gca aga gct g	200
13	1947 - 2051	105	II S3, II S4	16894	att tgg agg gag gag ttg gg	tca ctt tcc caa ctt tct gg	310
14	2052 - 2191	140	II S4, II S5	16894	cag aaa gtt ggg aaa tta gc	ttg aat tcc tct gaa gga c	250
15	2192 - 2264	73		16894	ctt gga gat gag aia ctg agc	cag gca ctt tca tct gtg ac	200
16	2265 - 2382	118	II S6	16894	tcc aca gct gca tct cca ag	acc ctc cct tga gcc cct	270
17	2383 - 2450	68	II S6	16894	cag tgg ttg ctt ttc ctg ac	ttg cca gag aaa cat tct cc	130
18	2451 - 2557	107		16894	tga aca aag att cca cgt gc	tcc agg agc cag ggt agc atc	170
19	2558 - 3367	810		16894	tag caa tgc tct aag tcc tg ccg agg aga acc gca aca a gc agc agg gag agc cgc agc	tgt ttc ctg agg aag tcc tc ggc atg acg tcc atg ctc tat cgt cat tct ggc gat tc	320 450 300
20	3368 - 3831	464		16894	ggt tct tt tca ttc act tgc gag aat agc ctt atc tgc ac	ttt cct ggc agt ctg agc tg cag tga tgg gag agc aga g	430 200
21	3832 - 3973	142		16894 / 34275	tgg gaa att tgg gag gga gc	tga ctt ccc cca ccc tgg tg	250
22	3974 - 4103	130	III S1	16894 / 34275	agc ctg tgg tct gag tgg ac	tag gaa ggg gtg tgc tct gtg	210
23	4104 - 4163	60	III S2, III S3	16894 / 34275	atc cac tgc tct ctt gct tt	gtg gtt ctc act tat aat ctg c	170
24	4164 - 4270	107	III S3	34275	tgg cct cat tgg ctt ccc tgc	aag agg aaa ccc ttg cga ag	250
25	4271 - 4370	100	III S4	34275	cta ccc aac ctg acc tct gc	aca tga taa ccc tga cag tc	220
26	4371 - 4531	161	III S5	34275	ctc atg ctc tct gtc aac tc	tgg ttc caa tgg gaa tgg gc	250
27	4532 - 4669	138		34275	ctg ctt ccc aag cag tct ag	tcc tgg ata gat ttc cag tc	300
28	4670 - 4871	207	III S6	34275	atg ttt taa agg aca gat gg	ttt ccc tgc ccc att cct ttg c	280
29	4872 - 5036	165	IV S1	34275	ctc tgc cgc tct cac cac tg	ttt atc agg tag agg cag g	250
30	5037 - 5147	111	IV S1, IV S2	34275	tcc caa gcc cat aqc tgg agc	tga ccc tgc tac tcc tgc ttc	180

31	5148 - 5231	84	IV S3	15496	act gtg cct cta aca tgc ac	aag tgc tgg ctc aag cag	250
32	5232 - 5348	117	IV S4	15496	tct gtg agt ggt gac agc tc	gtc acc tgt ctt ctc agc	240
33	5349 - 5414	66	IV S5	15496	tgg aag gac tct ggc acg tg	gga ggc tct ggg aac ctt ag	250
34	5415 - 5530	116		15496	aga agc cac tgg agg aat ggc	att atc aga gca ggt ccc ctt c	250
35	5531 - 5681	151	IV S6	15496	tcc gag tct ctg att tct cc	aga cgg ccc tca cag tgt c	210
36	5682 - 5809	128	IV S6	15496	tcc att ccc tcg gtc tct gc	ctg act gaa cct gtg aga c	350
37	5810 - 5906	97		15496	tgt gaa ccc att gcc tgc a	tgg gaa tga ctg cgc ttg c	200
38	5907 - 6012	106		15496	atg cct ggg aat gac tgc	tgt cac gcc tgt ctg tgc	200
39	6013 - 6120	108		15496	tga cac cca ggc agg cag	tct gtc ctg gtg gat tgg atc	200
40	6121 - 6221	101		15496	tgg gtg agc tca ccc tgt	tcc ccc tgg tga cat gca agc	200
41	6222 - 6331	110		15496	gtc cac aca ctg ctc tct gc	aca ctc cac ctc cct ggc	320
42	6332 - 6470	139		15496	gcc agg gag gtg gag tgt	ggg tcc ttc cac cgc aac	550
43	6471 - 6584	114		15496 / 30762	caa ctc ccc aat ggc tc	cct acc cag tgc aga gtg agg	350
44	6585 - 6620	36		15496 / 30762	tct gtg tgc acc atc cca tg	aag gat tgg gct cca tgg ag	200
45	6621 - 6807	187		15496 / 30762	gtt ggt gct agc tgc tga c	ctt tct tct tcc tta gtg tc	330
46	6808 - 7061	254		15496 / 30762	gtg tgc tgt ctg acc ctc ac	agc ctg ggg tca ctt gca gc	320
47	7062 - UTR	~350		/ 30762	cct ttt ttt caa ttt tct tgt ag	tgg ggc ctg ggt acc tcc da	280

Note. Sizes of exons and PCR products are given in basepairs;
 domains of protein are indicated according to Stea et al., 1995.

Table 2 Polymorphisms in coding sequence of the invented gene

<i>Location</i>	<i>Nucleotide change</i>			<i>Frequency</i>	<i>Consequence</i>
exon 4	nt 854	G - A	Thr ₁₉₃	0.02	-
exon 6	nt 1151	A - G	Glu ₂₉₂	0.07	-
exon 8	nt 1457	G - A	Glu ₃₉₄	0.38	-
exon 11	nt 1635	G - A	Ala ₄₅₄	0.02	Ala ₄₅₄ - Thr (A454T)
exon 16	nt 2369	G - A	Thr ₆₈₈	0.12	-
exon 19	nt 3029	G - A	Glu ₉₁₈	0.07	-
exon 23	nt 4142	T - C	Phe ₁₂₈₉	0.22	-
exon 46	nt 6938	T - C	His ₂₂₁	0.46	-
exon 47	nt 7213	(CAG),	3'UTR	=	-

Note. Frequency as observed in 100 control chromosomes; = Seven alleles of (CAG)_n were observed in the range between n=4 to n=14, with a heterozygosity value of 0.75.

Table 3 Mutations of the invented gene in families with FHM or EA-2

Disease	Family	Location	Domain	Nucleotide change		Consequence
FHM	It-II	exon 4	I S4	nt 850	G - A	Arg ₁₉₂ - Gln (gain of Sfcl site) R192Q
FHM	US-P	exon 16	P-segment	nt 2272	C - T	Thr ₆₆₆ - Met T666M
FHM	UK-B	exon 17	II S6	nt 2416	T - C	Val ₇₁₄ - Ala (gain of Bovl site) V714A
FHM	NL-A/US-C	exon 36	IV S6	nt 5706	A - C	Ile ₈₁₁ - Leu (gain of MnlI site) I1811L
EA-2	CAN-191	exon 22	III S1	nt 4073	deletion C	frameshift (loss of NlaIV site) STOP ₂₉₄
EA-2	CAN-26	intron 24	splice site	nt 4270-1	G - A	AC/gt - AC/at (loss of BsaAI site) aberrant splicing

Table 4. Parental transmission of migraine for 36 unrelated Dutch families.

<i>parents</i>	<i>N</i>	<i>offspring</i>	<i>N</i>	<i>affected N(%)</i>	<i>ratio*</i>
heathy father x migraine mother	51	daughters	72	48 (66.7%)	2.3:1
		sons	72	21 (29.2%)	
migraine father x healthy mother	18	daughters	26	17 (65.4%)	2.5:1
		sons	15	4 (26.7%)	

* ratio of proportion affected sons/proportion affected daughters